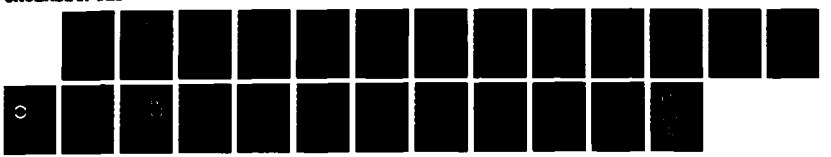
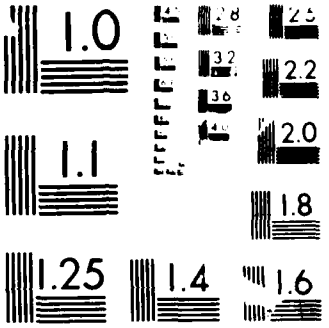
AD-A187 959 THE ELECTROFUSION MECHANISM IN ERYTHROCYTE GHOST MEMBRANES(U) JEROME H HOLLAND LAB ROCKVILLE MD A E SOMERS 30 OCT 87 N00014-87-K-0199 1/1

UNCLASSIFIED F/G 6/3 NL





MICROCOPY RESOLUTION TEST CHART

CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No 0704 0188

RT SECURITY CLASSIFICATION: (U)	1b RESTRICTIVE MARKINGS: NA
RITY CLASSIFICATION AUTHORITY: NA	DISTRIBUTION / AVAILABILITY OF REPORT: Distribution unlimited
ASSIFICATION DOWNGRADING SCHEDULE: NA	
RMING ORGANIZATION REPORT NUMBER(S): NA	MONITORING ORGANIZATION REPORT NUMBER(S): NA

E OF PERFORMING ORGANIZATION: American Red Cross	6b OFFICE SYMBOL (If applicable): NA	7a NAME OF MONITORING ORGANIZATION: Office of Naval Research
6c ADDRESS (City, State, and ZIP Code): Jerome H. Holland Laboratory, 15601 Crabbs Branch Way, Rockville, MD 20855		7b ADDRESS (City, State, and ZIP Code): 800 N. Quincy Street, Arlington, VA 22217-5000
8a NAME OF FUNDING SPONSORING ORGANIZATION: Office of Naval Research	8b OFFICE SYMBOL (If applicable): ONR	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: N00014-87-K-0199
8c ADDRESS (City, State, and ZIP Code): 800 N. Quincy Street, Arlington, VA 22217-5000		10 SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO	PROJECT NO	TASK NO	WORK UNIT ACCESSION NO
61153N	RR04108	4414708	

11 TITLE (Include Security Classification)

(U) The Electrofusion Mechanism in Erythrocyte Ghost Membranes

12 PERSONAL AUTHOR(S)

Arthur E. Sowers

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Annual	FROM 2/87 TO 10/87	October 30,1987	20

16 SUPPLEMENTARY NOTATION

NA

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	*Electroporation, Electrofusion, Membrane, Bioelectromagnetics, Mechanisms, Erythrocytes
08			

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Electrofusion is the method by which two parallel and close-spaced biological membranes can be fused into one membrane with an electric field pulse. Results of our experiments indirectly suggest that electropores are either not involved in the fusion mechanism or, if they are, then additional factors and processes are also involved. Results of our experiments directly and clearly suggest not only that electroosmosis takes place in the vicinity of electropores but that membrane mixing and contents mixing indicators actually measure different parts of the fusion process. Also shown is the finding that parts of the fusion process are reversible and have a time scale similar to that found for some naturally-occurring fusion processes.

20 DISTRIBUTION AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED-UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	(U)

22a NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c OFFICE SYMBOL
Dr. J. A. Majde	202/696-4055	ONR

DD Form 1473, JUN 86 Previous editions are obsolete SECURITY CLASSIFICATION

INTRODUCTION AND BACKGROUND

Electrofusion is the name given to the induction of membrane fusion by the use of electric field pulses. It is significant for three reasons: i) it is a clean system for studying electric field-membrane interactions; ii) electrofusion is a practical in vitro method of inducing fusion for biotechnology applications such as genetic engineering and obtaining monoclonal antibody-secreting hybrids; and iii) a fundamentally new way to study the molecular mechanism of membrane fusion.

This report is an abrieviated version of a paper which will become a chapter (Sowers and Kapoor, 1988a) in a monograph to come out in late 1988 which will be devoted to electroporation and electrofusion in cell biology (Plenum Press). This book is presently being edited by E. Neumann, the author of this report, and C. Jordan. The book will cover primarily applications as well as theoretical and experimental studies dealing with electroporation, electrofusion, and dielectrophoresis. Most of the writings cover work done in the last ten years while some (including the present author's) work emphasizes the most recent findings and includes data not yet published and an interpretation which more coherently integrates previously made experimental observations.

This report emphasizes those parts of the chapter paper which cover the reporting period. Our earlier reviews cover the electrofusion mechanism in erythrocyte ghosts (Sowers and Kapoor, 1987a), as well as emphasize the role of electropore collisions in the fusion mechanism (Sowers and Kapoor, 1987b), and the molecular mechanism of electrofusion before (Sowers and Kapoor,1988b) and after (Sowers and Kapoor, 1988a) we discovered the significance of contents mixing events (see below).

We have been studying electrofusion for the last 5 years. During that period we have selected an idea model membrane, designed and constructed appropriate instrumentation, developed and perfected assays, defined levels of reproducibility and instrumentation tollerances, and characterized a variety of phenomena related to both electrofusion and electroporation. The last 1-1/2 years have witnessed a considerable increase in our understanding of electrofusion and electroporation.

The electrofusion protocol as originally presented (Zimmermann, 1982) involves the application of one or more electric field pulses to membranes held in close contact (the direction of the field must be perpendicular to the plane of the two membranes at the location where they are in contact). In contrast to this membrane-contact-before-pulse (referred to hereafter as the "contact-first" or CF) protocol, we have discovered that fusion will occur even if pulses are applied to membranes while they are in random position and then brought into contact afterwards (Sowers, 1983; Sowers, 1984; Sowers, 1985b; Sowers, 1986a; 1986b). We refer to this procedure as the "pulse-

first" or PF protocol to both name and contrast this protocol with the original CF protocol. PF electrofusion has now been reported from another laboratory to occur in chinese hamster ovary cells (Teissie and Rols, 1986). Fusion by this protocol has been observed later by others in some membrane systems (Bates, Saunders, and Sowers, 1987; Zimmermann et al., 1984) but not fully recognized as possibly revealing a fundamentally different fusion mechanism or acknowledged as an important variation in the normal protocol. Our discovery of PF fusion was surprizing since it was contrary to and not predictable from the previously published explanations for the fusion mechanism (Zimmermann, 1982;1986). This discovery is significant because it may shed light not only on how membranes fuse in nature but may be exploitable in drug-delivery applications.

Elucidating the mechanism of electrofusion is made more difficult by the fact that the mechanism of fusion in any membrane system is still poorly understood. Also, the possibility that electropores are part of the intermediate structure is part of some hypotheses concerning the electrofusion mechanism (see below), however no experimental data has been presented by other laboratories to support this contention. Results from our lab suggest that electropore induction does not appear to corellate with electrofusion. We found that pulse treatment of membranes in suspension led to a transient permeabilization in which some combination of the pore lifetime, diameter, and numbers decreased as the ionic strength of the medium was increased (Sowers, 1986a). This is in agreement with what has been generally found as the effect on electropore induction with changes in ionic strength (see reviews). However, when the same membranes were aligned into close contact (i.e. the PF protocol was used), an increase in ionic strength caused an increase in fusion yield (see Fig. 4). This is opposite to what would be expected if there were a simple relationship between pore formation and fusion. The situation as we now understand it is more complex than this because pulse-induced pores open to large sizes and then immediately reclose within a 100-200 msec time scale (Sowers and Lieber, 1986). However, the reclosing is incomplete and a residual pore of about 1 nm diameter is formed. Consequently when the PF protocol is used, the electropores which would have to be participating in the fusion process would have residual rather than peak diameters. It is difficult to see how such a small pore could be involved in membrane fusion.

METHODS:

Our studies of the electrofusion mechanism utilized the human erythrocyte ghost membrane as a model membrane (Dodge et al, 1963). We utilized dielectrophoresis (Pohl, 1978) to induce close membrane-membrane contact. Dielectrophoresis was significant for our studies because it is a mild, reversible, and non-chemical effect with undetectable or nearly undetectable side effects on cells.

Our two electrode chamber (Fig. 1) was designed to be easy to construct, disposable, and permit continuous observation of the membranes by light microscopy. The electric field induced in a given chamber is calculated by dividing the voltage applied to the electrodes by the distance between electrodes. An additional chamber was designed and contructed (Fig. 1) for separate experiments in which the membrane alignment direction and the direction of the electric field pulse could be varied (see below).

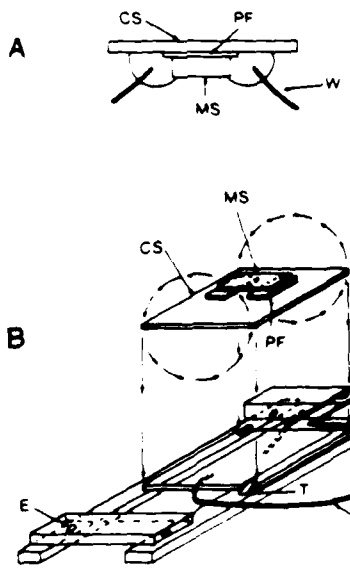
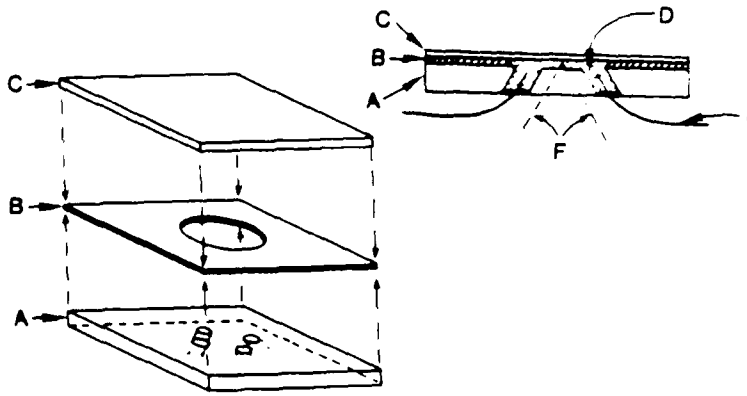


FIGURE 1. Fusion slide. (A) Fusion chamber made by heat sealing of a microslide (MS) with parafilm (PF) in a dry mounting press to a coverslip (CS). (B) fusion chamber mounted with tape (T) in a frame made from glass strips cut from a standard microscope slide and cemented together with epoxy cement (E). Wire electrodes (W) are anchored to the frame with epoxy cement.



Chamber to permit axis of electric field lines from AC used align membranes to be parallel or perpendicular to axis of electric field lines from DC fusion-inducing pulses (see Fig. 2). Upper view is cross section; lower view is exploded perspective. Plexiglas (R ... and Hass Co., West Hill, Ontario, Canada) sheet (2.4 mm thickness) in which four electrode holes (1.6 mm diameter) converge at ... angle to center (center-to-center hole spacing: 3.2 mm). Chamber is formed by Plexiglas sheet (A), Parafilm (American Can Co., Greenwich, CT) sheet (B) with hole (5.5 mm diameter) to surround ... limits of electrode holes, forms a gasket between Plexiglas sheet (A) cover slip (C). Seal is made by application of heat to the Parafilm. Membrane suspension (D) is added to chamber through one of the electrode (E) holes. Lines (F) indicate path of light from ... condenser.

Fig. 1. Left is from Sowers (1984), right is from Sowers (1986).

Our present pulse generator is based on a type 5557 mercury vapor thyratron as a switch element (Fig. 2). We also designed a square wave pulse generator utilizing mercury-wetted relays (Fig. 3) and used it to obtain preliminary data (see below) by which a comparison could be made with exponentially-decaying pulses.

A storage screen oscilloscope was used to continuously monitor the pulse waveform characteristics (voltage and decay half-time) and the effect of the equivalent load resistance of the chamber.

Since, our previous studies showed that phase optics can underestimate fusion yields (Sowers, 1984), we decided that fusion needs to be scored on the basis of more rigorous criteria. Since fusion of membranes permits intermixing of all laterally

mobile membrane components, fusion is revealed if some of the ghosts which are aligned into pearl chains are labeled with the fluorescent lipid analog, 1,1'-dihexadecyl-3,3,3',3'-tetramethylindo carbocyanine perchlorate, (DiI).

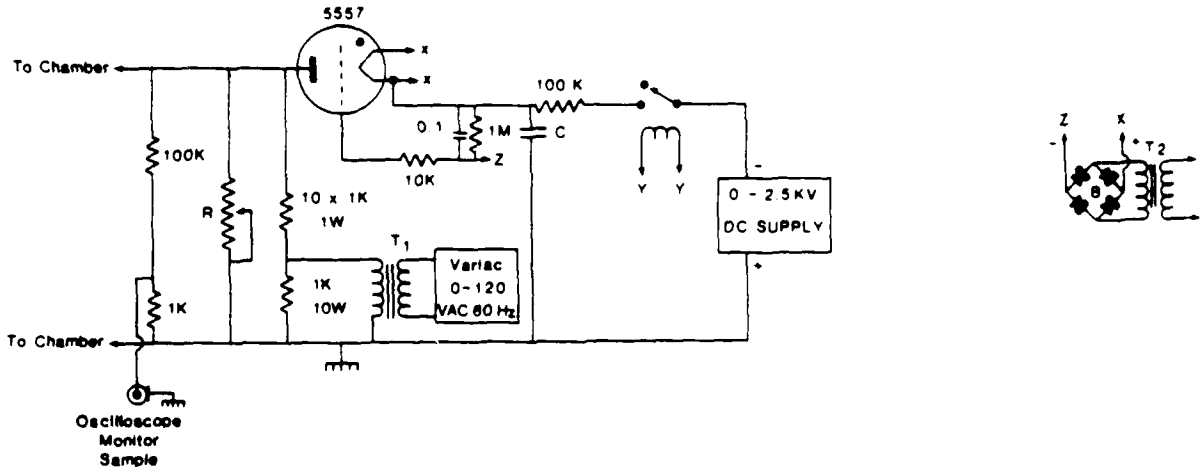


Fig. 2. Essential details of pulse generator circuit which uses a type 5557 mercury vapor thyratron. See Sowers and Kapoor, 1988a for further details.

Fusion thus
results in a time dependent movement of fluorescence (from the labeled membrane to one or more unlabeled membranes) at rates which are consistent with the lateral diffusion coefficient for lipids (Sowers, 1985a).

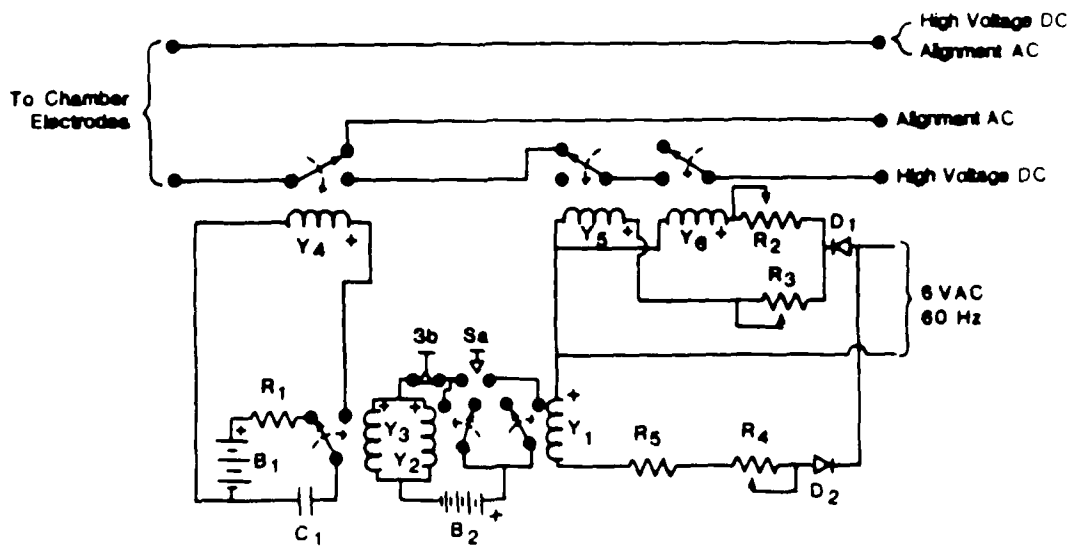


Fig. 3. Circuit for pulse generator which provides single square wave pulses with a magnitude up to several hundred volts. See Sowers and Kapoor, 1988a for further details.

Also, the formation of a lumen between two originally unfused but close-spaced membranes will permit the aqueous diffusion of a soluble molecule (we used 10 kD FITC-Dextran) previously placed within the enclosed space of one membrane to the interior space of the adjacent membrane. These two post-fusion possibilities are commonly recognized as operational criteria for fusion and are referred to as "contents mixing" (for FITC-Dextran) and "membrane mixing" (for DiI), respectively.

We have also found that fusion can be <u>detected</u> indirectly without fluorescent-tagged molecular labels by virtue of the fact that pulses will induce attachment between membranes in close contact. Indeed, using the CF protocol and turning off the AC-induced dielectrophoresis after a pulse treatment this attachment appears as a fraction of membranes which do not return to random posistions. Proof of fusion in these membranes was demonstrated when all (>99%) attached membranes showed laterally diffusing DiI.

The use of fluorescent tagged lipid-soluble molecules (to label the membrane) or fluorescent tagged water-soluble molecules (to label the cytoplasmic compartment), is necessary to positively demonstrate fusion. A fusion event is scored if a pulse results in the movement of the label to an unlabeled membrane in a pearl chain adjacent to a labeled membrane. Fusion yield was calculated by counting the number of unfused labeled membranes, N_u, counting the number of occurrences (=fusion events) in which the label moved to at least one adjacent but originally unlabeled membrane, N_f, and calculating $N_f/(N_f + N_u)$. This number (always between 0 and 1) was converted to percent by multiplying it by 100.

EXPERIMENTAL OBSERVATIONS AND DISCUSSION

In the light of reports (eg. Sukharev et al., 1985) and discussions at scientific conferences that low voltage long pulses will also permeabilize and may fuse membranes as well as high voltage short pulses, we modified our pulse generator and indeed demonstrated that these pulses would also fuse membranes using the CF protocol (Fig. 4). This led to a significant extention (Sowers and Kapoor, 1988a) of our previous studies about the range of field strengths and decay half-times that a pulse can have and still induce pores and fusion. In general, fusion yield was found to be proportional to field strength, decay half-time, and ionic strength but there is some reciprocity between the "duration" that current is flowing and the field strength of the pulse. This applies also to the PF protocol at least over the more limited range of E and $T_{1/2}$ as previously explored (Sowers, 1986a; Sowers, 1987b).

Fig. 4 shows, however, that although use of lower pulse voltage and longer decay halftime will also lead to fusion, the <u>maximum possible</u> fusion yield will be <u>lower</u> and that the experimental control of the field strength and decay halftime will require

more accuracy in the pulse generator. This could be a source of high fusion yield variability reported by many investigators (Bates, et al., 1987).

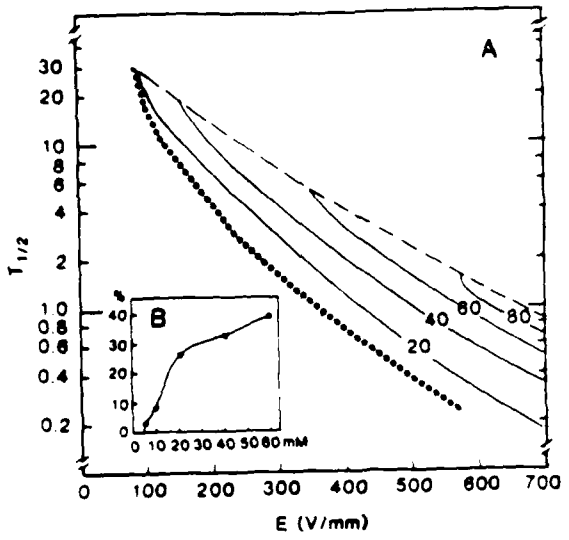


Fig. 4. Fusion yield (A) as induced with a single field pulse in erythrocyte ghosts as a function of electric field strength and decay half-time in 60 mM sodium phosphate buffer (pH 8.5) and (B) as a function of buffer (=ionic) strength (inset). Fusion yield measured using DiI as the fluorescent label and using the CF protocol (see text). Pulse parameters: above dashed line lead to fragmented membranes; below dotted line lead to no effects; between dotted line and dashed line lead to both fusion (isofusion contours in percent fusion yield) and electroporation (as revealed by simultaneous loss of mw = 10 kD FITC-Dextran from cytoplasmic compartments with application of a single pulse). Ghosts were isolated and labeled as previously described (Sowers, 1984).

Although square wave pulses and exponentially-decaying pulses are commonly used, no studies have compared the effects of the two waveforms on the same membrane system under the same conditions. During the preliminary study conducted with the mercury-wetted relay pulse generator we obtained data (Table 1) suggesting no major qualitative differences for the two waveforms.

Our observations indicate that fusion nearly always coincides, within human perception, with the application of a pulse when the CF protocol is used. Conversely, fusion rarely, if ever, occurs at any finite time after a pulse. Consequently, when the CF protocol is used we think of the pulse-induced membrane change which leads to fusion as involving a transient or short-lived structural change. On the other hand, when the PF protocol is used, fusion occurs only at the moment the membranes are brought into contact and never afterwards. This clearly calls for a long-lived fusogenic modification of the membrane which allows fusion to be triggered upon contact.

We have also found that human erythrocyte ghosts gradually loose their ability to be electrofused as they age during storage. This requires that fusion experiments be conducted no later than one day after membrane preparation (data not shown).

The conventional notion that rigorous demonstration of fusion is satisfied if and only if evidence of both membrane continuity and communication between compartments can be obtained. In the case of electrofusion of erythrocyte ghosts this

Table I. Effect of pulse* waveform on fusion yields (percent)

		waveform							
				square					
		exp.-decay.		40 usec		120 usec		400 usec	
buffer		protocol		protocol		protocol		protocol	
strength:	N:	CF	PF	CF	PF	CF	PF	CF	PF
LUMEN-PRODUCING FUSION PRODUCTS ONLY:									
20 mM	3	24	1	0	0	0	0	7	0
	10	35	2	21	10	23	11	4	3
60 mM	3	35	22	0	0	0	0	0	0
	10	76	46	0	0	11	7	4	0
ALL FUSION PRODUCTS (non-lumen plus lumen):									
20 mM	3	nd	nd	10	2	36	1	71	8
	10	nd	nd	51	20	65	17	94	35
60 mM	3	nd	nd	10	1	47	0	45	11
	10	nd	nd	40	4	61	13	89	50

*-Buffer was sodium phosphate and the pulse field strength was 700 v/mm. N is number of pulses, applied at the rate of two per second. The rectangular chamber, the preparation of the human erythrocyte ghosts used in the assay, and the exponentially-decaying waveform pulse generator were as previously described (Sowers, 1984). Exponentially-decaying pulses had a decay halftime of 0.6 msec. When the PF protocol was used, the time interval between pulses and induction of membrane-membrane contact was 15 sec.

is apparent through the use of FITC-dextran and DiI as indicators of, respectively, contents mixing and membrane mixing. The conventional expectation would then be that each fusion event would require both one contents mixing event and one membrane mixing event to occur in each pair of membranes.

However, measurements of CF-protocol fusion events under a variety of conditions showed significantly more occurrences of fluorescence movement into unlabeled membranes when FITC-dextran was used than when DiI was used as a label (Fig. 5)(Sowers, 1988a; 1988b). The analysis of this discrepancy requires some background discussion.

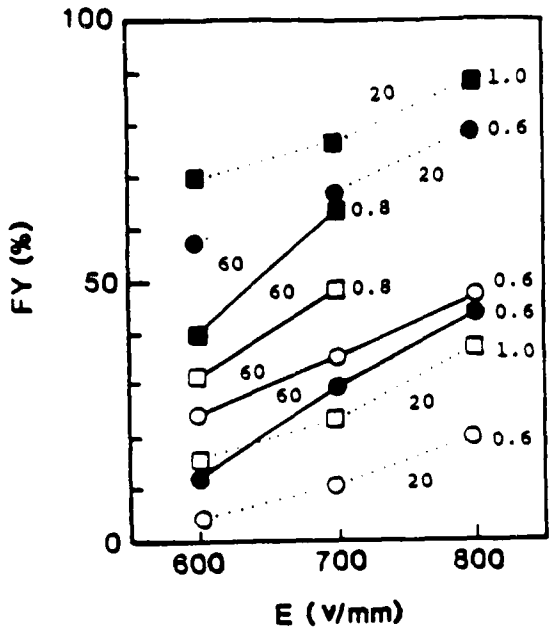


Fig. 5. Fusion yield (%) as function of field strength, E, decay halftime (0.6 or 1.0 msec), buffer (=ionic) strength (20 or 60 mM sodium phosphate), and event indicator (solid = FITC-Dextran, open = DiI). pH = 8.5 and CF protocol used in all cases.

NOTE: raise E, $T_{1/2}$, get increase in both FYs, but raise ionic strength and get increase in DiI-based FY but a decrease in FITC-based FY!

The interpretation of this apparent discrepancy was aided by three additional observations. First, a large difference in pulse-induced permeabilization between the cathode-facing and the anode-facing hemispheres was reported from three laboratories (Mehrle et.al, 1985; Rosignol et al., 1983; and Sowers and Lieber, 1986). Second, we observed that the fusion-inducing pulse caused the FITC-Dextran in mixutures of labeled and unlabeled membranes in pearl chains to move to adjacent unlabeled membranes predominantly (70-95% of all events) towards the negative electrode and rarely towards the negative direction or in both directions (Sowers, unpublished). These movements were usually complete within 20-100 msec. Lastly, we have observed that the application of electric pulses induces a shape change in the erythrocyte ghosts regardless of whether they are in pearl chains or in random positions in the suspension (Fig. 6)

Nearly all erythrocyte ghosts in sodium phosphate buffer (pH 8.5) will be perfect spheres at or below a buffer concentration of about 15 mM. From 15 mM to about 25-30 mM, the geometry will convert smoothly and completely to an irregular collapsed sphere or discoid-like shape. Discoids will be predominant above about 30 mM. When pulses are applied one at a time to suspensions of ghosts which show the irregular shape, a "wave" propagates from the positive-facing hemisphere towards the negative-facing hemisphere in a fraction of a second (Sowers, 1984). Additional pulses causes a stepwise transformation of the shape until the perfect sphere geometry is attained. If ghosts start

with the perfect sphere geometry, the pulses have no effect (except see below).

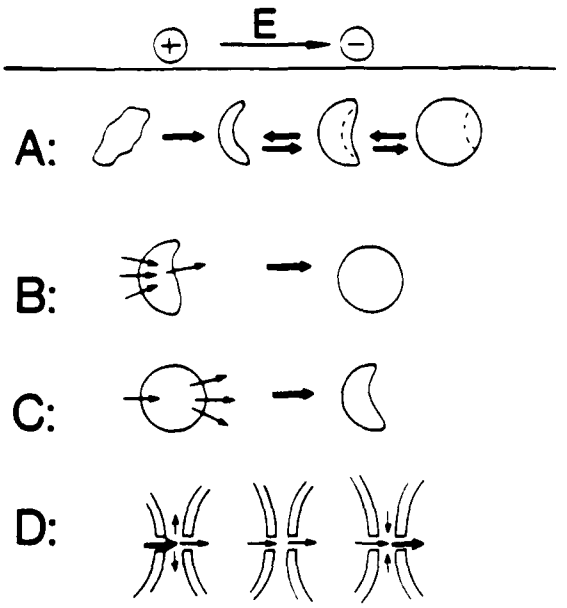


Fig. 6. Interpretation of pulse-induced erythrocyte ghost shape with respect to participation of electroosmosis (cf. Fig. 10): (A) shape change as shown in Fig. 8. Pulse application in direction shown causes irregular shapes (left) to become spherical (right) by passing through a cup-shaped intermediate (middle two steps). Process is reversible for a finite number of cycles (see text). (B) the pulse-induced volume increase (as occurs with low ionic strengths and short decay half-time pulses) can take place if a greater influx occurs in the positive-facing hemisphere than outflux at the negative-facing hemisphere. (C) the pulse induced decrease in volume (as induced at low ionic strength with longer decay half-time pulses) as would occur with a greater outflux at negative-facing hemisphere. (D) Effect of uneven electroosmosis flow through concentric pore pairs: Left - net inflow to inter-membrane space would push membranes appart. Middle - no effect. Right - net removal of medium from inter-membrane space might bring membranes closer together favoring fusion.

The amount of shape change per pulse seems to be greatest when pulse field strength and pulse decay half-time coincided with that needed for fusion. Longer decay half-time pulses above a field strength threshold caused fragmentation of the membrane and pulses with much shorter decay half-time or much lower voltage caused neither fusion nor shape change. We also noticed that at low ionic strength (20 mM) application of pulses with field strengths and decay half-times just under the threshold for fragmentation caused spherical-shaped ghosts to convert to stomatocytes with the concave surface facing the negative electrode. If ghosts were not spherical, then pulse application caused them to end in stomatocytes instead of spheres. Conversely, once the ghosts were in the stomatocyte shape, application of a pulse with a shorter decay half-time caused the shape to return to that of a spherocyte (Fig. 6).
By switching back and forth between long and short decay half-time on alternate pulses it was possible to change shape alternately and reversibly at least several times before the reversibility was lost.

The above three observations were puzzling until we asked two fundamental questions about fusion events reported by FITC-Dextran movement from labeled to adjacent unlabeled ghosts.

First, we wondered if the cytoplasmic compartments in a pearl chain which became contiguously labeled following a pulse really did represent fused membranes. This was tested by simply applying pulses to membranes in pearl chains and then turning off the AC immediately after the pulse. The result was that linear and continuous groups of two, three, and sometimes more, labeled ghosts sometimes separated into individual labeled ghosts and diffused apart while other linear groups remained connected to one another in linear groups. The only change was that the axes of the linear groups became disoriented with time by Brownian motion.

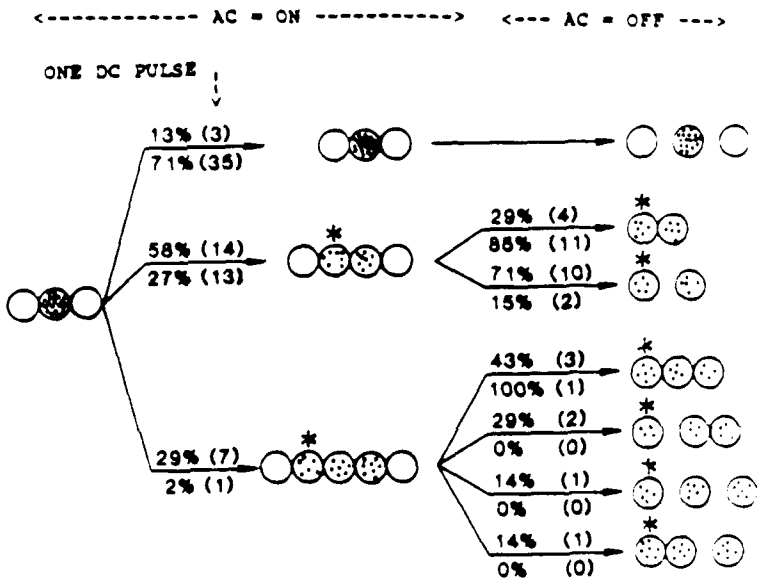


Fig. 7. Experiment to determine how many contents-mixing events are actually fusion events. Mixtures of FITC-Dextran labeled and unlabeled ghosts are aligned with AC into pearl chains (left). Then a single fusion-inducing pulse is applied which leads to a variety of unlabled ghosts to become labeled with FITC-Dextran diffusing from originally labeled (*) ghosts therby indicating a contents mixing event (middle). Turning off the AC after the pulse (right) shows that some groups of membranes which became labeled remained attached to each other (= actually fused) while other groups diffused appart (= actually unfused). Over 99% of attached membranes represent fused membranes as proven in separate experiments using DiI to indicate membrane connection. Numbers in parentheses indicate number of events while numbers with percents are percentage of total. Numbers above/below arrows are for 20 mM/60 mM, respectively, in sodium phosphate at pH 8.5.

Observation by phase optics of pulse treated DiI-labeled ghosts in pearl chains followed immediately by turning off the AC afterwards showed that a fraction of the membranes returned to random positions and another fraction which remained attached to one another in linear groups although the axes of these groups eventually became disoriented by Brownian motion (Sowers, 1984). However, in fluorescence optics at least 99 % of the groups that contained one DiI-labeled ghost also showed DiI laterally diffusing into unlabeled membranes. Taking the laterally diffusing DiI as evidence that fusion had taken place led to the conclusion that every point of pulse-induced attachment between membranes actually represented fusion of those membranes.

When combined with the observation of pulse-induced FITC-Dextran movements followed by either separation or non-separation after the AC was turned off, then all of the separation

occurrences actually must have represented unfused membranes. Fused membranes would be attached through a connection made between originally separate membrane areas which would permit the lateral diffusion of the lipid soluble label, DiI. Similarly, such attachments would be detected when the AC was turned off after a pulse. Thus the contents mixing event reported by FITC-dextran movement must be interpreted to represent the induction by the pulse of a transient high conductance (permeability) channel which is a fusion precursor or intermediate stage. Since the induction of this channel is transient and is not accompanied by significant loss of fluorescence to the external medium, then it must have a short lifetime (on the order of 20-50 msec). The induction of this channel must also be reversible since it does not always lead to fusion.

The second question was prompted by the observation of predominant movement of FITC-dextran towards the negative electrode. The clue to the answer to this question was found when ghost membranes were aligned into pearl chains with AC and then, just before applying a pulse, the AC is turned off for a few seconds until the membranes start separating from each other as Brownian motion starts pushing them back to random positions. Alternate viewing by phase and fluorescence optics permitted an FITC-Dextran labeled and nearby (1-3 um) unlabeled ghost to be on or near the electric field pulse axis to be located. Focus and immediate application of the pulse allowed the movement of FITC-Dextran fluorescence to be followed using by low light level video microscopy out of the negative-facing hemisphere of the labeled ghost, formation of a transient cloud outside both ghosts, and, lastly, accumulation of fluorescence in the originally unlabeled membrane (i.e. 1-3 um apart).

The interpretation of this observation is that the soluble marker must enter the cytoplasmic compartment of the originally unlabeled ghost through a transient permeabilization in the positive-facing hemisphere. Entry through the permeabilization which we know occurs in the negative hemisphere (Sowers and Lieber, 1986) is unlikely since the concentration of FITC-Dextran is high in the transient cloud of fluorescence between the two ghosts but probably very low in the vicinity of the negative-facing hemisphere.

This observation then suggests that both hemispheres are similarly, although not necessarily identically, permeabilized. This observation can be made consistent with the other reports (Mehrle, et.al., 1985; Rossignol, et al., 1983; Sowers and Lieber, 1986) if electroosmosis (Balasubraminian and McLaughlin, 1982) occurs at each electropore (Fig. 10). In both of the other reports, the molecule or ion which is used to trace the permeabilization starts from a location <u>outside</u> the cytoplasmic compartment and moved <u>inward</u> with the pulse treatment while in our study the FITC-dextran label started from <u>inside</u> the cytoplasmic compartment and moved <u>outward</u> with the pulse treatment.

The reversible pulse-induced shape change therefore must be a manifestation of an electroosmosis-based aqueous flow into the cytoplasmic compartment at the positive-facing hemisphere and out of the cytoplasmic compartment at the negative-facing hemisphere. Change in shape from a collapsed sphere to a perfect sphere must occur by virtue of a volume increase. This can only occur with a net influx of aqueous medium which would mean a greater influx than outflux. When outflux is greater, then stomatocytes are formed and this is consistent with the fact that fusion does not occur.

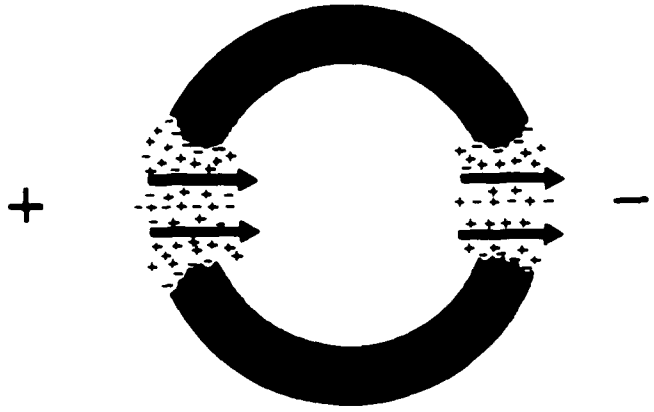


Fig. 8. Effect of electroosmosis (see text) in vicinity of conventional pores at center of both hemispheres of a membrane in an electric field. Field direction is left to right. Excess of protons and cation counter ions over anions near the negative-charged surface of the membrane would experience an electrophoretic force towards the negative electrode. However, flow of medium towards negative electrode would dominate. Hence net flow would be into sphere at anode-facing hemisphere and outward at cathode-facing hemisphere.

Integration of our data into existing electrofusion hypotheses:

At the present time hypothetical electrofusion mechanisms fall into two categories: a) electropore hypotheses, and b) point defect hypotheses.

Fusion mechanisms involving electropore: Electropores have been invoked as part of fusion mechanisms at least partly because much is known about their properties and they are induced by the same pulses which induce fusion. Membrane electropore induction occurs when induced transmembrane potentials are above a poorly defined threshold. Electroporation of membranes by electric field pulses has been known for some time and has been recently reviewed by workers in several laboratories (Tsong, 1983; Baker and Knight, 1983; Neumann, et al., 1982; Knight and Scrutton, 1986). The simplest explanation for this permeability increase is that the pulse induces pores or holes in the membrane although little is known about the relationship between the characteristics of the external field pulse and the shape, numbers, effective diameter, lifetime, and location of these pores. Their effective diameter can be probed by the use of labeled molecules with known diameter, however.

Although much is known about electropores there is little information available to predict their effective diameter, number, lifetime, shape, or location. From numerous experimental studies, however, resealing in electropores has been found to have the following characteristics. Resealing is: a) faster at higher temperature, b) near zero at low temperatures, and c) faster or sooner at high ionic strengths, and d) complete in artificial phospholipid bilayers (Teissie and Tsong, 1981) but incomplete and or slow in erythrocyte membranes (Serpersu et al., 1985; Schwister and Deuticke, 1985). Electropores appear to have a maximum radius which is inversely related to ionic strength.

Electropores are important becaue they are part of two similar electrofusion hypothetical mechanisms (Fig. 9). Pilwat et al. (1981) pictured the electrofusion mechanism as a process in which the electric field pulses induce short-lived pairs of electropores as an intermediate membrane structure. The individual lipid molecules continually change in position to form a fused membrane. In the original proposal (Pilwat et al.,1981) the space surrounded by the pore pair is proposed to be transiently occupied by a cloud of water-solvated lipid molecules. The dispersed form that these molecules take is evidently necessary to help bridge the gap between each of the close-spaced but originally separate membranes. However, the basis for expecting lipids to be dispersed is unclear; calculations indicate that heating is insignificant (Pastushenko, 1983). Pohl et al (1984) apparently favored a similar sequence but included the possibility that one of the two pores opened first while Pilwat et al. (1981) pictured the mechanism as involving concentric pairs of pores which open simultaneously. The mechanism presented by Pohl et al. (1984) was unfortunately not accompanied by illustrative diagrams.

The Dimitrov and Jain (1984) proposal is a four-step mechanism. To bridge the space between the membranes, they propose that the pores develop a "flare" so that the hydrophobic free edges of the pores can touch each other and fuse with each other. The two pores do not include the feature of the cloud of lipid molecules presented by Pilwat et al.,(1981). Sugar et al. (1987) proposed a model for fusion which includes the propagation of a crack-like defect as part of the fusion process. We consider their model as a variation on the Dimitrov and Jain (1984) model, rather than the Pilwat et al (1981) model, since concentric pore pairs are involved but, in contrast, do not include a cloud of dispersed lipid molecules.

Fusion involving point defects in membranes: Stenger and Hui (1986) conducted an interesting freeze-fracture electron microscopy study of electrofusion in human erythrocytes. Indeed, their electron micrographs show both point defect-like structures and flap-like structures but their interpretation of the micrographs is that membrane fusion occurs as a consequence of the formation of the point defects in the membrane (Fig. 9). The flap-like structures appear to be reversibly inducible structures that should have electropore properties even though they do not

have the cylindrical geometry implied by either the Pilwat et al (1981) model or the Dimitrov and Jain (1984) models. It is important to consider this model as one which does not depend on electropores as part of the intermediate structure. More will be said below about this study.

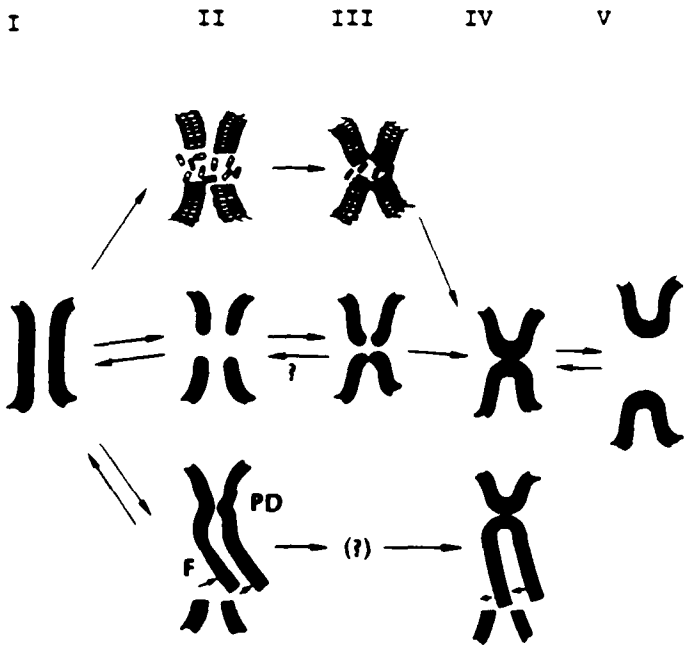


Fig. 9. Multi-step (I - V) models for the mechanism of electrofusion: Fusion process procedes from unfused but close-spaced membranes (I) at left to fused membrane (V) with expanding lumen at right. Upper path (addapted from Pilwat et al.,1981): fusion starts with pulse-induced concentric pore formation (II) (pores filled with dispersed lipid molecules) and proceding to fusion product (IV) by passing through an intermediate stages (II and III) in which the lipid molecules change position in an orderly but apparently continuous forward process which reforms two membranes into one. Middle path (addapted from Dimitrov and Jain, 1984): induction of concentric pairs of pores (II) proceeds to a stage in which the edges of the pores become flared (III) before ending with a fusion product (IV). Lower path (adapted from Stenger and Hui,1986): Fusion involves pulse-induced point defects (PD) and pulse-induced "flaps" (F) which follow separate fate paths. The point defects lead to fusion while the flaps reseal after providing a large cytoplasmic-cytoplasmic compartment conductance for soluble molecules to move from one compartment to another. Configurations at stage II and III of the pathways represent molecular configurations which are at beginning and end of the lifetime of the intermediates. The intermediate structure in the model represented by the lower path was arbitrarily placed at stage II since the authors' description of the process did not permit unambiguous assignment. Note that all pathways can permit contents mixing events. However, contents mixing events without membrane connections would make the path to at least stage II and possibly stage III reversible (note added arrows in reverse direction). It is unlikely that the upper path can be reversible if the process is self-completing as originally implied (Pilwat et al.,1981) since formed membranes are thermodynamically stable and intermediates will be expected to be unstable. Lumen expansion (IV -> V) is reversible as previously reported (Sowers, 1984).

Relationship between our experimental observations and models. Our interpretation (see above) that the pulse-induced FITC-Dextran movements represent the reversible induction of an intermediate structure favors the idea that fusion involves a multistep and reversible process. This is not as compatible with the Pilwat et al., (1981) mechanism, which appears to be a

continuous and irreversible process, as with the Dimitrov and Jain (1984) model. The Dimitrov and Jain model has two substeps (1. concentric pore pairs, and 2. concentric pore pairs with flares) in the interval between unfused and fused membranes. Our FITC-Dextran movements cannot distinguish between these substeps but does appear to reflect the notion that there is some relationship between electroporation and fusion since the number of fusion events is always lower than the number of contents-mixing events. However, the Stenger and Hui (1986) model physically separates the fusion-initiating structure (point-defects) from the membrane structure (the "flaps") which would be responsible for contents-mixing. Their interpretation does allow for concentric-pore pairs, but they have a flap-like structure. Their study did not include experiments to determine the effect of a change in ionic strength although our experimental results suggest that an increase in ionic strength may lead to a decrease in the number or size or lifetime in their population of flaps and a possible increase in the number of their point defects (assuming a one-to-one relationship between point defects and fusion events).

Implications of the long-lived fusogenic state. Our experimental observation of fusion in membranes if they are treated with many electric pulses before they are brought into close contact was contrary to the widely accepted protocol (CF) which called for membrane-membrane contact to be established before and maintained during the pulse treatment. This experimental fact demonstrated that the pulses induce a long-lived fusogenic state in the membranes. Also, this experimental observation makes it impossible for concentric pore pairs to be formed upon pulse treatment as single entities. Hence the mechanism must involve additional unknown mechanistic details. One hypothetical possibility is that as two pulse-treated membranes are aligned into close contact, a head on collision could take place between a pore on one membrane and a pore on a second membrane. Thus a concentric pore-pair could be formed which would then be followed by a squence which would end in a fusion event. Our preliminary calculations, however, indicate that this would be too unlikely to account for the observed fusion yields (Sowers, 1986a; Sowers, 1986b; 1987a; 1987b; Sowers and Lieber, 1986). On the other hand, the calculations are sensitive to the assumptions and work is in progress to make refinements in both the measurements and the theory behind the calculations. Never the less, the idea that a concentric pore pair fusion intermediate might be assembled from individual pores implies that electropores could be fusogenic sites.

We have observed that the fusogenic areas induced by the multiple pulse treatment using the PF protocol are relatively small and that the fusogenic property does not laterally diffuse away from the areas of the membrane where it is induced and diffuse into areas where it is not induced (Fig. 10)(Sowers 1986a, Sowers, 1987b). A similar observation was made by Teissie and Blangero (1984) but for use of the CF protocol on cultured cells. In this sense the fusogenic property does not behave as if

it were composed of membrane pores made totally out of lipid molecules and are totally surrounded by lipid molecules. Such pores would laterally diffuse in the plane of the membrane at rates comparable to laterally mobile membrane components (Sowers, 1985a; Sowers and Hackenbrock, 1981).

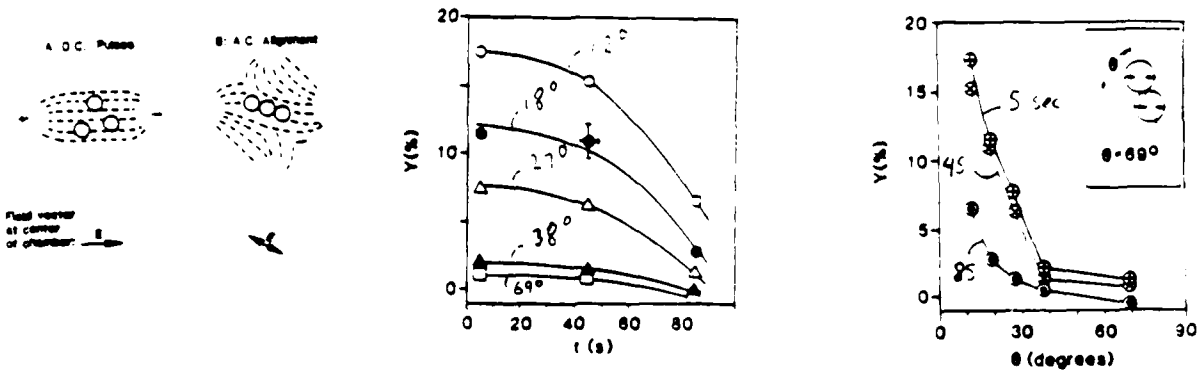


Fig. 10. Synopsis of theory and results from experiment to determine whether the pulse-induced long-lived fusogenic state has components in the membrane which are laterally mobile (from Sowers, 1987b). Left: a four electrode chamber (see Fig. 1 this report) was used. Dirrect current pulses are fed into two oposing electrodes (part A) and then alternating current is fed to all four electrodes in the proper almplitude and phase relationship to cause the alignment axis to be variable with respect to the pulse field axis, Middle: Fusion yield (%) as a function of time (sec) between application of pulses and induction of membrane-membrane contact. Angles are given in degrees of arc. Right: Fusion yield (%) as function of angle between pulse field direction and alignment axis direction for the three time intervals between pulse application and induction of membrane-membrane contact. Note that for the 5 sec and 45 sec time intervals and at angles between 10-35 degrees, and for the 85 sec time interval between angles of 12 and 18 degrees, that the slopes of all the lines are identical. Therfore, the fusogenic state is essentially immobile on a time scale compatible with all conventional measures of lateral mobility.

5. SUMMARY-SIGNIFICANCE

Our characterization of the electrofusion mechanism has begun to lead to a greater understanding of the way electric field forces interact with a natural plasma membrane to induce the connection (fusion) of two parallel and close-spaced erythrocyte ghost membranes.

1. Two fundametally different protocols (the PF and the CF) can induce membrane fusion. This suggests that a possibly very different mechanism is involved.

2. The long-lived fusogenic state is a membrane property which is laterally immobile. This can be interpreted in terms of a single domain (over a large area) with a continuously variable fusogenicity or many discrete perturbations (each with point or small areas) of the membrane which are locally-induced domains

linked to membrane proteins and/or the cytoskeletal system. If electropores are involved then they must be physically linked to the immobile domains and/or proteins. However all electropores as previously conceived are made of lipids and would be free to diffuse laterally in the plane of the membrane. This is incompatible with the data.

3. Fusion induced by the CF protocol can now be interpreted to involve an experimentally detectable intermediate stage which has a short lifetime and its induction is reversible.

4. Electroosmosis appears to accompany the electrofusion process but whether it directly or indirectly influences the fusion process is not yet clear. Electroosmosis, however, may significantly increase the complexity of analyzing and characterizing electropore induction.

5. While the induction of the fusion intermediate is an apparently reversible process, higher ionic stregth promotes the formation of fusion products from intermediate states and diminishes the probability of reforming the unfused membranes. This finding reveals fusion as a process clearly involving at least three experimentally identifiable steps.

Regardless of whether the PF or CF protocol is used, the data suggest at least that there is a more complex relationship between electric field treatment of membranes and either electropore induction or membrane fusion. While the two effects are both consequences of an electric field pulse they may be unrelated to one another.

The low light level video data indicate that electrofusion may have some features in common with fusion in natural systems. This is especially relevant since naturally-occurring fusion in at least some membrane systems also appears to be reversible and have a time scale (Breckenridge and Almers, 1987; Fernandez, et al., 1984; and Zimmerberg et al., 1987) which is compatible with that for electrofusion in erythrocyte ghosts.

References

Baker, P.F., and Knight, D.E., 1983, High voltage techniques for gaining access to the interior of cells: Application to the study of exocytosis and membrane turnover, in Methods in Enzymology, 98:(pt. L) 28-37.

Balasubraminian, A., and McLaughlin, S., 1982, Electroosmosis at the surface of phospholipid bilayer membranes, Biochim. Biophys. Acta. 685:1-5.

Bates, G., Saunders, J., and Sowers, A.E., 1987, Electrofusion: Principles and applications, in: Cell Fusion, (A.E. Sowers, ed), pp. 367-395, Plenum Press, New York.

Breckenridge, L.J., and Almers, W., 1987. Final steps in exocytosis observed in a cell with giant secretory granules. Proc. Natl. Acad. Sci. U.S.A. 84:1945-1949.

Dimitrov, D.S., and Jain, R.K., 1984, Membrane stability,

Biochim. Biophys. Acta 779:437-468.
Dodge, J.T., Mitchell, C., and Hanahan, D.J., 1963, The preparation and chemical characterization of hemoglobin-free ghosts of human erythrocytes. Arch. Biochem. Biophys. 100:119-130.
Fernandez, J.M., Neher, E., and Gomperts, B.D., 1984, Capacitance measurements reveal stepwise fusion events in degranulating mast cells, Nature 312:453-455.
Knight, D.E., and Scrutton, M.C., 1986, Gaining access to the cytosol: The technique and some applications of electropermeabiliziation, Biochem. J. 234:497-506.
Mehrle, W., Zimmermann, U., and Hampp, R., 1985, Evidence for asymmetrical uptake of fluorescent dyes through electro-permeabilized membranes of Avena mesophyll protoplasts, FEBS let. 185:89-94.
Neumann, E., Schaefer-Ridder, M., Wang, Y., and Hofschneider, P.H., 1982, Gene transfer into mouse lyoma cells by electroporation in high electric fields, EMBO J. 1:841-845.
Pastushenko, V.F., 1983, Pore heating during electrical breakdown of bilayer lipid membranes, Biologicheskie Membrany 1:176-181.
Pilwat, G., Richter, H.-P., and Zimmermann, U., 1981, Giant culture cells by electric field-induced fusion, FEBS Let. 133:169-174.
Pohl, H.A., 1978, Dielectrophoresis, Cambridge Univ. Press, London.
Pohl, H.A., Pollock, K., and Rivera, H., 1984, The electrofusion of cells, Int. J. Quant. Chem: Quant. Biol. Symp. 11:327-345.
Rossignol, D.P., Decker, G.L., Lennarz, W.J., Tsong, T.Y., and Teissie, J., 1983, Induction of calcium-dependent, localized cortical granule breakdown in sea-urchin eggs by voltage pulsation, Biochim. Biophys. Acta 763:346-355.
Schwister, K., and Deuticke, B., 1985, Formation and properties of aqueous leaks induced in human erythrocytes by electrical breakdown, Biochim. Biophys. Acta 816:332-348.
Serpersu, E.H., Kinosita, Jr., K., and Tsong, T.Y., 1985, Reversible and irreversible modification of erythrocyte membrane permeability by electric field, Biochim. Biophys. Acta 812:779-785.
Sowers, A.E., 1988a, Does electrofusion involve the induction of a distinct intermediate stage? (submitted)
Sowers, A.E., 1988b, Evidence for a fusion intermediate in electrofusion, Biophys. J. (submitted)
Sowers, A.E. 1987a, Cell Fusion, Plenum Press, New York.
Sowers, A.E., 1987b, The long-lived fusogenic state induced in erythrocyte ghosts by electric pulses is not laterally mobile, Biophys. J. 52: (in press).
Sowers, A.E., 1986a, A long-lived fusogenic state is induced in erythrocyte ghosts by electric pulses, J. Cell Biol. 102:1358-1362.
Sowers, A.E., 1986b, Long-lived fusogenic membrane sites induced by electric field pulses are not free to diffuse laterally in the plane of the membrane, Biophys. J. 49:132a.
Sowers, A.E., 1985a, Movement of a fluorescent lipid label from a labeled erythrocyte membrane to an unlabeled erythrocyte membrane following electric field-induced fusion, Biophys. J. 47:519-52[illegible].

Sowers, A.E., 1985b, Electric field-induced membrane fusion in erythrocyte ghosts: evidence that pulses induce a long-lived fusogenic state and that fusion may not involve pore formation. Biophys. J. 47:171a.

Sowers, A.E., 1984, Characterization of electric field-induced fusion in erythrocyte ghost membranes, J. Cell Biol. 99:1989-1996.

Sowers, A.E., 1983, Red cell and red cell ghost membrane shape changes accompanying the application of electric fields for inducing fusion, J. Cell Biol. 97:179a.

Sowers, A.E., and Hackenbrock, C.R., 1981, Rates of lateral diffusion of intramembrane particles: Measurements by electrophoretic displacement and rerandomization, Proc. Natl. Acad. Sci. U.S.A. 78:6246-6250.

Sowers, A.E., and Kapoor, V.,1988a, The mechanism of electrically-induced fusion in erythrocyte membranes, in: Electroporation and electrofusion in cell biology (E. Neumann, A.E.Sowers, C.A. Jordan, eds), Plenum Press, New York (in press).

Sowers, A.E., and Kapoor, V., 1988b, The mechanism of erythrocyte ghost fusion by electric field pulses, in: Procedings of the International Symposium on Molecular Mechanisms of Membrane Fusion, (S.Ohki, ed.), pp. (in press), Plenum Press, New York.

Sowers, A.E., and Kapoor, V., 1987a, The electrofusion mechanism in erythrocyte ghosts, in: Cell Fusion, (A.E. Sowers, ed), pp. 397-408, Plenum Press, New York.

Sowers, A.E. and Kapoor, V., 1987b, Fusogenic Membrane Alterations induced by electric field pulses, in: Mechanistic approaches to interactions of electromagnetic fields with living systems, (M. Blank and E. Findl, eds), pp.325-337, Plenum Press, New York.

Sowers, A.E., and Lieber, M.L., 1986, Electropores in individual erythrocyte ghosts: diameters, lifetimes, numbers, and locations, FEBS Let. 205:179-184.

Stenger, D.A., and Hui, S.W., 1986, Kinetics of ultrastructural changes during electrically induced fusion of human erythrocytes, J. Memb. Biol. 93:43-53.

Sugar, I.P., Forster, W., and Neumann, E., 1987, Model of cell electrofu Membrane electroporation, pore coalescence and percolation, Biophys. Chem. 26:321-335.

Sukharev, S.I., Popov, S.V., Chernomordik, L.V., Abidor, I.G., 1985, A patch-clamp study of electrical breakdown of cell membranes, Biologicheskie Membrany 2:77-86.

Teissie, J, and Blangero, C., 1984, Direct experimental evidence of the vectorial character of the interaction between electric pulses and cells in cell electrofusion, Biochim. Biophys. Acta 775:446-448.

Teissie, J., and Rols, M.P., 1986, Fusion of mammalian cells in culture is obtained by creating the contact between cells after their electropermeabilization, Biochem. Biophys. Res. Comm. 140:258-264.

Teissie, J., and Tsong, T.Y., 1981, Electric field induced transient pores in phospholipid bilayer vesicles, Biochemistry 20:1548-1554.

Tsong, T.Y., 1983, Voltage modulation of membrane permeability and energy utilization in cells, Bioscience Reports 3:487-505.

Zimmerberg, J., Curran, M., Cohen, F.S., Broderick, M., 1987, Simultaneous electrical and optical measurements show that membrane fusion precedes secretory granule swelling during exocytosis of beige mouse mast cells, Proc. Natl. Acad. Sci. U.S.A. 84:1585-1589.

Zimmermann, U., 1986, Electrical breakdown, electropermeabilization and electrofusion, Rev. Physiol. Biochem. Pharmacol. 105:175-256.

Zimmermann, U., 1982, Electric field-mediated fusion and related electrical phenomena, Biochim. Biophys. Acta 694:227-277.

Zimmermann, U., Buchner, K.-H., and Arnold, W.M., 1984, Electrofusion of cells: recent developements and relevance for evolution, in: Charge and Field Effects in Biosystems, (M.J. Allen and P.N.R. Usherwood, eds), pp. 293-318, Abacus Press, Normal, Illinois.

***

DISTRIBUTION LIST

Bioelectromagnetics Program

Annual, Final and Technical Reports (one copy each)

INVESTIGATORS

Dr. W. R. Adey
J. L. Pettis Memorial VA Hospital
11201 Benton Street
Loma Linda, CA 92357

Dr. Stephen Cleary
Virginia Commonwealth University
Box 694 - MCV Station
Richmond, VA 23298

Dr. C. C. Davis
Department of Electrical Engineering
University of Maryland
College Park, MD 20742

Dr. Carl Durney
Department of Electrical Engineering
University of Utah
Salt Lake City, UT 84112

Dr. Kenneth R. Foster
Bioengineering Department
University of Pennsylvania
Philadelphia, PA 19104

Dr. Reba Goodman
Columbia University
630 West 168th Street
New York, NY 10032

Dr. A. W. Guy
Department of Rehab. Medicine, RJ-30
University of Washington
Seattle, WA 98195

Ms. Carol Jordan
SAIC, 1710 Goodridge Drive
P.O. Box 1303
McLean, VA 22102

Dr. Adrianus J. Kalmijn
Scripps Institution of Oceanography
Ocean Research Division, A-020
La Jolla, CA 92093

Dr. Bruce Kleinstein
Information Ventures, Inc.
1500 Locust Street
Philadelphia, PA 19102

Dr. Raphael Lee
Department of Electrical Engineering
and Computer Science
Massachusetts Institute of Technology
Cambridge, MA 02139

Dr. S. M. Lindsay
Department of Physics
Arizona State University
Tempe, AZ 85287

Dr. Thomas C. Rozzell
National Research Council, FG 424
2101 Constitution Avenue
Washington, DC 20418

Dr. Asher Sheppard
Research Service 151
J. L. Pettis Memorial VA Hospital
Loma Linda, CA 92357

Dr. Betty Sisken
Wenner-Gren Research Laboratory
University of Kentucky
Lexington, KY 40506

Dr. Arthur E. Sowers
American Red Cross
Holland Laboratory
15601 Crabbs Branch Way
Rockville, MD 20855

Dr. Shiro Takashima
Bioengineering Department
University of Pennsylvania
Philadelphia, PA 19104

Dr. Watt W. Webb
Department of Applied Physics
Cornell University
Ithaca, NY 14853

Annual Final and Technical Reports (one copy each except as noted)

ADMINISTRATORS

Scientific Officer, Biophysics Program
Code 1141SB
Office of Naval Research
800 N. Quincy Street
Arlington, VA 22217-5000

Program Manager
Biological/Human Factors Division
Office of Naval Research, Code 125
800 N. Quincy Street
Arlington, VA 22217-5000

Program Manager, Molecular Biology
Code 1141MB
Office of Naval Research
800 N. Quincy Street
Arlington, VA 22217-5000

Program Manager
Support Technology Directorate
Office of Naval Research, Code 223
800 N. Quincy Street
Arlington, VA 22217-5000

Administrator (2 Copies; enclose DTIC Form 50)
Defense Technical Information Center
Building 5, Cameron Station
Alexandria, VA 22314

Administrative Contracting Officer
ONR Resident Representative
(address varies--obtain from business office)

Annual and Final Reports Only (one copy each)

DOD ACTIVITIES

Commander
Chemical and Biological Sciences Division
Army Research Office, P.O. Box 12211
Research Triangle Park, NC 27709

Director
Department of Microwave Research
Walter Reed Army Institute of Research
Washington, DC 20307-5001

Directorate of Life Sciences
Air Force Office of Scientific Research
Bolling Air Force Base
Washington, DC 20332

Program Manager
Radiofrequency Radiation Program
U.S. Air Force School of Aerospace Medicine
Brooks Air Force Base, TX 78235

Library
Armed Forces Radiation Research Institute
Bethesda, MD 20814-5145

Final and Technical Reports Only (six copies each)

Director, Naval Research Laboratory
Attn: Technical Information Division, Code 2627
Washington, DC 20375

END

DATE

FILMED

FEB.

1988